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Direct reprogramming of terminally differentiated B cells into erythroid lineage

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ABSTRACT

Hematopoietic progenitors have been shown to retain plasticity and switch lineages by appropriate stimuli. However, mature blood cells hardly showed such differentiation plasticity. In this paper, we tried to reprogram mature B cells into erythroid lineage by expressing various hematopoietic transcription factors. Among various factors, GATA-1, SCL together with CCAAT/enhancer binding protein (C/EBP) α turned out to be a minimal set of factors that efficiently reprogrammed terminally differentiated mature B cells into erythroid lineage, as evidenced by colony forming assays and erythroid-specific gene expressions. This study sets an avenue to generate autologous erythrocytes from peripheral B cells.

1. Introduction

Differentiation of hematopoietic cells is a sequential process of cell fate decision dictated by the lineage-specific transcription factors. For example, differentiation of granulocyte monocyte progenitors (GMPs) from common myeloid progenitors (CMPs) critically depends on the expression of CCAAT/enhancer binding protein (C/EBP) α [1,2]. On the other hand, GATA-1 is critical for the development of megakaryocytes and erythroid cells [3–5]. These lineage-specific transcription factors possess strong lineage instructing capacities by activating gene expression programs specific to the respective lineage.

It is now widely accepted that hematopoietic progenitors retain developmental plasticity and they can be reprogrammed by appropriate lineage-converting stimuli. It was reported that common lymphoid progenitors (CLPs) and pro-T cells could be converted into granulocyte/monocyte (GM) lineage by enforced cytokine signals, suggesting that immature lymphoid cells retain latent myeloid differentiation potential [6]. It was subsequently shown that ectopic expression of lineage specific transcription factors such as GATA-1 or C/EBPα could redirect immature hematopoietic progenitors to erythroid/megakaryocytic or myelomonocytic lineage, respectively [7–9]. We have also shown that conditional activation of C/EBPα could reprogram CLPs, megakaryocyte/erythroid progenitors (MEPs), and immature B and T cells into GM lineage [10]. These data suggest that overexpression of lineage-specific transcription factors can override the lineage commitment program and induce lineage switch. Moreover, further studies revealed that C/EBPα could reprogram terminally differentiated mature B cells into myeloid cells [8]. However, mature lymphocytes have never been reprogrammed into erythroid lineage.

Somatic cell reprogramming into induced pluripotent stem cells (iPSCs) is a strikingly powerful way to obtain a patient-specific cell source for regenerative medicine, and differentiation protocols for iPSCs to various tissue-specific cells are now under active investigation. In hematopoietic system, induction of mature blood cells such as erythrocytes or platelets from iPSCs is an attracting possibility to obtain autologous blood cells for transfusion. However, induction of mature blood cells from iPSCs generally requires elaborative culture process, and implies a risk of teratoma formation by contaminated undifferentiated iPSCs when they are applied to patients. Therefore, direct reprogramming is considered to be more feasible and practical way for obtaining specific type of tissue cells for clinical application compared to using iPSCs or embryonic stem cells (ESCs).

In this study, we attempted to reprogram terminally differentiated mature B cells into erythroid lineage, and to define minimal set of transcription factors required for erythroid reprogramming. We found that the combination of GATA-1, SCL and C/EBPα was sufficient to reprogram mature B cells into erythroid lineage. This is the first report to demonstrate the reprogramming of mature B cells into erythroid lineage and opens up an avenue to generate autologous erythrocytes from easily obtainable, peripheral B cells.

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2. Materials and methods

2.1. Mice

C57BL6/J (Ly5.2) mice were from Japan CLEA Inc. (Tokyo, Japan). C/EBPα-ER transgenic (Tg) mice were described previously [10]. Mice from 8 to 12 weeks old were used in all experiments. All mice were bred and maintained in specific pathogen-free environment, and all animal experiments were carried out according to the guidelines for animal use issued by the Committee of Animal Experiments, Keio University School of Medicine.

2.2. Reagents

4-Hydroxytamoxifen (4-HT, Sigma–Aldrich) was dissolved in ethanol (EtOH) at 5 mM and was used at the final concentration of 1 μM. All cytokines were purchased from R&D Systems.

2.3. Antibodies

The following monoclonal antibodies were used for flow cytometric analysis: FITC-anti-B220 (Biolegend), PE-anti-CD19 (BD Pharmingen), FITC-anti-CD45.2, PE-anti-CD45, PE-anti-CD3 (eBioscience), FITC-anti-Gr1, and PE-anti-CD11b (BD Biosciences).

2.4. Plasmids

Retrovirus vector expressing full-length murine GATA-1 fused to the mutated ligand-binding domain of the mouse estrogen receptor (pMXs-IRES-GFP (IG)/GATA-1-ER) and pMSCV-GFP/GATA-2 is a generous gift from Dr. Ezoe, Osaka University. pMXs-IG/GATA-1 was from Dr. Nakano, Osaka University. pMYs-IG/Hes-1, pMYs-IG/SCL and pGCDNSan-IG/Evi-1 are generous gifts from Dr. Kurokawa, University of Tokyo. pMY-IG/C/EBPα-ER was described previously [10].

2.5. Production of retrovius

Retrovirus plasmids were transiently transfected into PLAT-gp cells using Fugene 6 (Roche Diagnostics) according to the manufacturer's protocol. Medium was changed 24 h after transfection, and supernatants containing retrovirus were collected after another 24 h. To make stable retroviral producer lines, PLAT-E cells were infected in the presence of 10 μg/ml polybrene (Sigma–Aldrich) with retrovirus produced by PLAT-gp cells, and 2–3 days later, GFP^{high} PLAT-E cells were sorted using MoFlow cytometer (Beckman Coulter). Sorted PLAT-E cells were expanded in the culture, and their culture supernatant was used as high-titer retroviral supernatants.

2.6. Isolation and retroviral transduction of murine mature B cells

Spleen cells were obtained from C57BL6/J (Ly5.2) mice or C/EBPα-ER Tg mice, and mononuclear cells were separated by density-gradient centrifugation using Lymphoprep (Axis-Shield PoC AS). Mature B cells were enriched by magnetic cell sorting using MACS B cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated B cells were stained with anti-B220-FITC and anti-CD19-PE antibodies, and analyzed by FACS Caliber (BD bioscience) for their purity. The purity of B cells was more than 95% in all experiments.

Purified B cells were stimulated in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 10 μg/ml Lipopolysaccharide (LPS; Sigma–Aldrich), 0.1 μg/ml anti-CD40 (BD Biosciences) and 10 ng/ml IL-4 (Peprotech). Retrovirus infection was performed using Retronectin (Takara Bio) according to the manufacturer's protocol.

2.7. Colony assay

B cells infected with retrovirus (1×10^5 cells/dish) were cultured in MethoCult GF M3434 (Stem Cell Technologies) supplemented with 10 ng/ml of human (h) thrombopoietin (TPO), 10 ng/ml of mouse (m) granulocyte macrophage-colony stimulating factor (GM-CSF), with or without 1 μM 4-HT. After 7–10 days of culture, number of colonies was enumerated under an inverted microscope. For the morphological examinations, colonies were picked, cytospun onto glass slides, and stained by Wright–Giemsa staining. Fluorescent images were examined and captured by laser confocal microscope (Olympus).

2.8. Extraction of DNA and RNA

Extraction of genomic DNA was performed using DNeasy Blood & Tissue Kit (Qiagen). Total RNA was extracted using Trizol reagent (Invitrogen), and then treated with RNase-free DNase I (Invitrogen) to remove contaminating genomic DNA. Messenger RNA (mRNA) was isolated using Micro-FastTrack 2.0™ mRNA Isolation Kit (Invitrogen).

2.9. PCR analysis

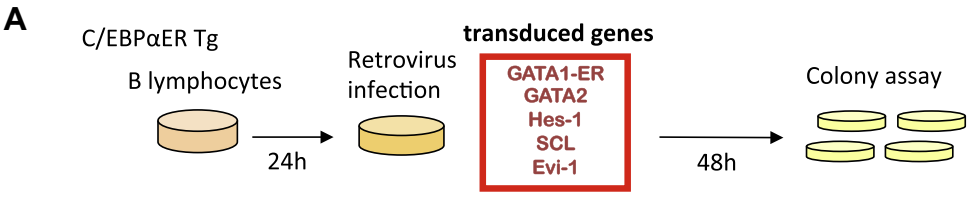
PCR analyses for rearrangements of immunoglobulin heavy chain genes were carried out as previously described [8] using Ex Taq-HS polymerase (TaKaRa). To examine the integration of transduced genes, PCR was performed using the primer sets listed in *Supplementary* Table 1. For RT-PCR, cDNA was synthesized with Super Script II reverse transcriptase (Invitrogen), and PCR was performed using Ex Taq-HS polymerase (TaKaRa). Primer sequences for lineage-specific genes and GAPDH were described previously [7].

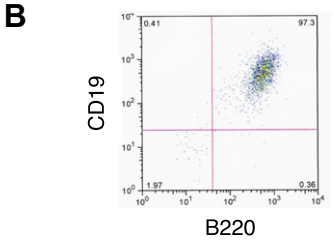
3. Results

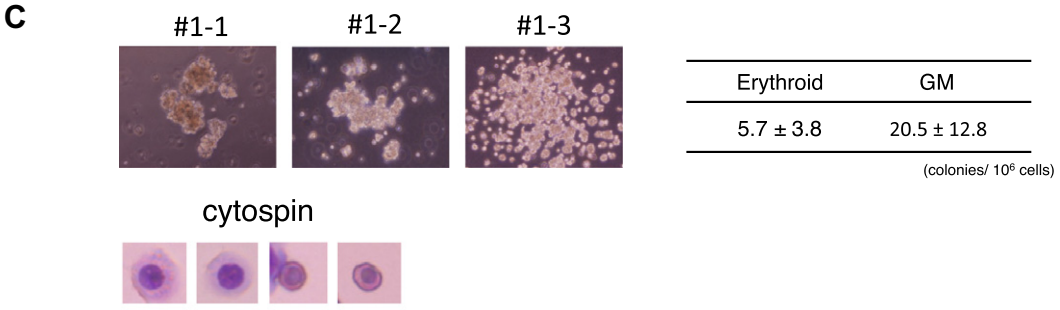
3.1. Mature B cells can be reprogrammed into erythroid-like cells by co-introduction of various transcription factors

To reprogram B cells into erythroid lineage, we tried to express transcription factors related to erythroid differentiation such as GATA-1 and SCL. In addition, we used transcription factors expressed in immature hematopoietic cells such as GATA-2, Hes-1 and Evi-1, hoping that they facilitate the reprogramming process (Fig. 1A). Of note, GATA-2 is highly expressed not only in immature hematopoietic cells, but also in MEPs [7], suggesting that it may be useful for erythroid conversion. Previous study has shown that C/EBPα is required for efficient reprogramming of mature B cells [11], and therefore we decided to include C/EBPα in reprogramming factors. For activating C/EBPα in B cells, we employed transgenic mice expressing conditional form of C/EBPα (C/EBPα-ER Tg) whose activity can be induced by 4-hydroxy tamoxifen (4-HT) [10]. We have also used inducible form of GATA-1 (GATA-1-ER) for the experiments. As noted in the discussion, however, weak activity of C/EBPα-ER or GATA-1-ER leaking under the non-induced condition was sufficient to induce erythroid conversion of B cells, and reprogramming efficiency as well as the phenotype of reprogrammed cells was not different between induced- and non-induced states. For this reason, we hereafter do not specifically mention the supplemental status of 4-HT unless otherwise required.

We purified splenic B cells from C/EBPα-ER Tg mice, and stimulated them with anti-CD40 and LPS. Activated B cells with over

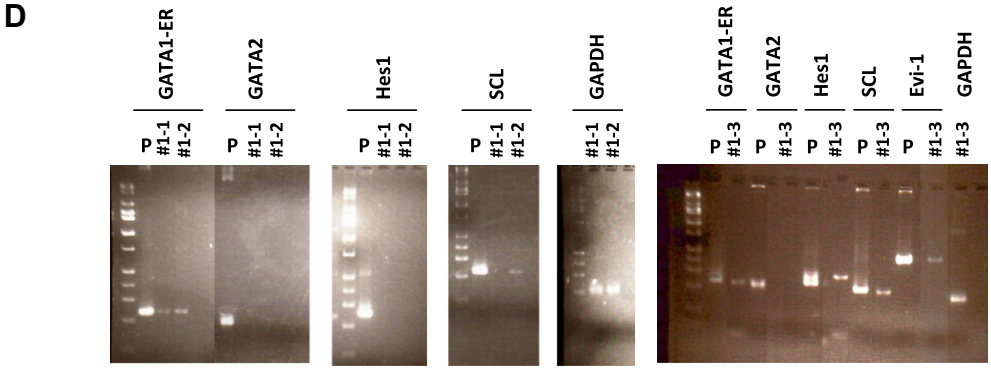






Erythroid	GM
5.7 ± 3.8	20.5 ± 12.8

(colonies/ 10^6 cells)



Integrated genes

	GATA-1	GATA-2	Hes1	SCL	Evi1
#1-1	+	-	-	-	-
#1-2	+	-	-	+	-
#1-3	+	-	+	+	+

Fig. 1. Reprogrammingof B cells from C/EBPα-ER Tg or WT mice into erythroid-like cells. (A) Schematic illustration of retrovirus transduction to B lymphocytes from C/EBPα-ER Tg mice and colony assay. (B) Purity of mature B cells used for retrovirus transduction. Expression of B220 and CD19 was assessed by flow cytometry. (C) Representative erythroid-like colonies on day 7–10 of cultures (upper panel. Original magnification: 20X) and the morphology of erythroid cells by cytospin preparation (lower panel. Wright-Giemsa staining. Original magnification: 400X). Numbers of erythroid-like (erythroid) and myeloid (GM) colonies generated are shown on the right (mean ± S.D., $n = 3$). (D) PCR analysis for integration of transduced genes. Sample numbers that correspond to those in (C) are shown on each lane. The primer sets used for PCR analysis are shown in *Supplementary* Table 1. P; positive control. (E) Rearrangements of IgH gene in erythroid-like colony converted from B cells. Polyclonal splenic B cells from C57BL6/J mice were used as a control (left). Representative results from the converted erythroid-like colony are shown on the middle and the right. A, B, C, D, E, F, G, DQ52, $DFSJ_{H4A}$ stand for the name of the primers set in the exons of V_H, D_H or J_H region of IgH gene as previously described [8]. Position of each primer is schematically presented at the bottom. Neg; negative control. (F) Reprogramming of wild-type B cells into erythroid lineage. Transduced genes are shown on the list on the upper panel. Morphologies of representative erythroid-like colonies formed in the presence or absence of 4HT are shown on the lower panel. Numbers of erythroid-like (erythroid) and myeloid (GM) colonies generated by this protocol are shown on the lower-right (mean ± S.D., $n = 6$).

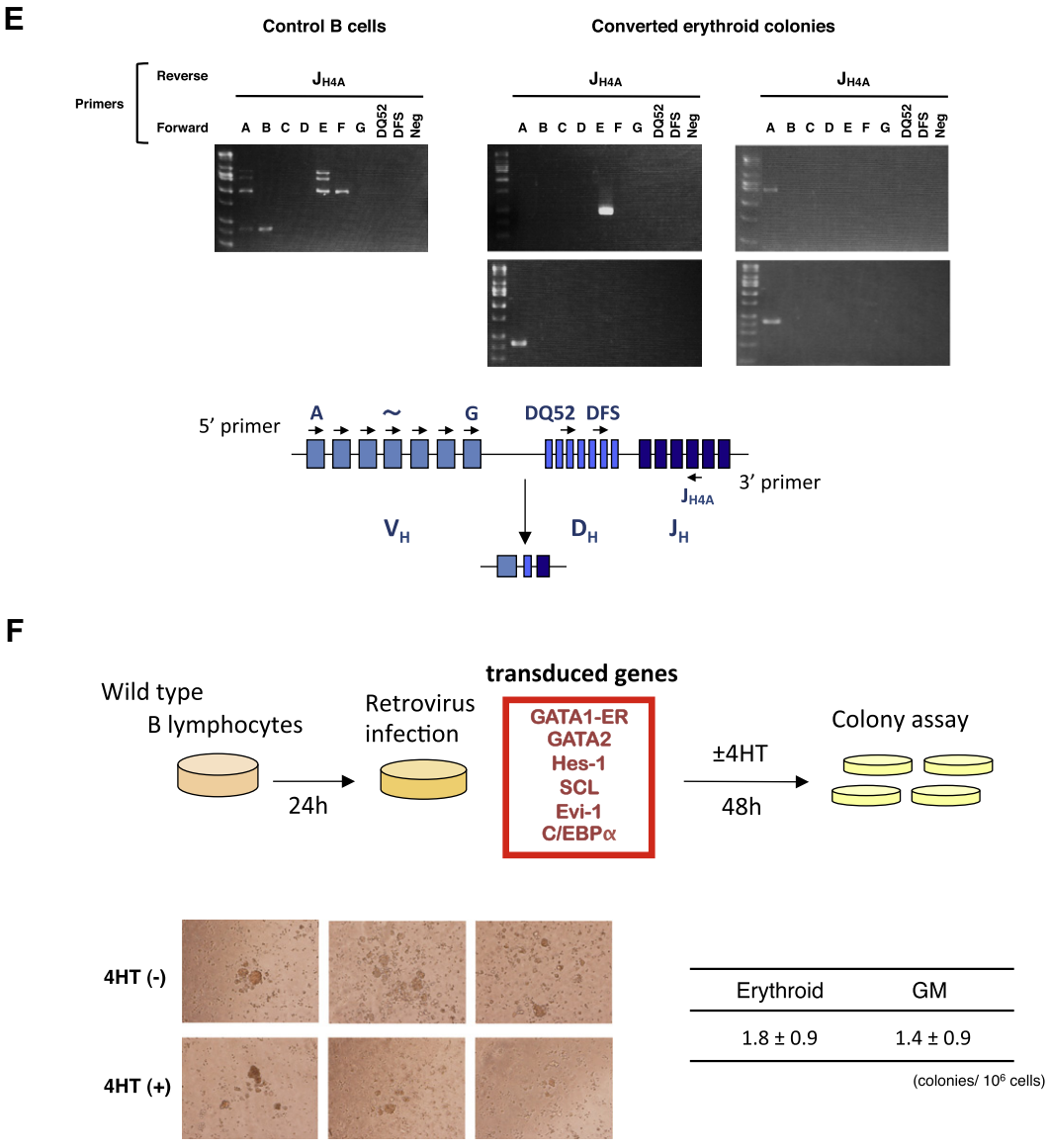


Fig. 1. (*continued*)

95% purity (Fig. 1B) were transduced with the above listed genes by retrovirus, and differentiation capacity of infected cells was assessed by colony assays. After 7-days of culture, erythroid-like colonies highly similar to burst forming unit-erythroid (BFU-E) showed up in culture plates (Fig. 1C). Examination of cellular morphology by cytospin preparation confirmed that the cells isolated from the colonies presented erythroid morphology (Fig. 1C). We picked up individual colonies and examined the integration of transduced genes by PCR (Fig. 1D). Of three colonies examined, GATA-1-ER was commonly integrated in all three clones, whereas SCL was integrated in two clones. One clone retained Hes-1 and Evi-1 transgenes in addition to GATA-1-ER and SCL. To check whether these erythroid-like colonies were converted from B cells, we examined the rearrangement of immunoglobulin heavy chain (IgH) genes by genomic PCR. As shown in Fig. 1E, rearrangement of IgH gene could be detected in the cells from the erythroid-like colony, showing that they originated from B cells. It is of note that all erythroid-like colonies were positive for GFP (data not shown), and mock-infected B cells formed very few erythroid colonies (*Supplementary* Table 2), suggesting that erythroid-like colonies were not derived from the contaminated erythroid precursors.

We have also tried reprogramming of splenic B cells from wild-type (WT) mice instead of C/EBPα-ER Tg mice by transducing the same set of transcription factors together with C/EBPα-ER using retrovirus (Fig. 1F). As expected, conversion of B cells into erythroid-like colonies could be achieved by this combination of factors either with or without 4HT.

These data suggest that terminally differentiated B cells can be reprogrammed into erythroid lineage by introducing GATA-1-ER, SCL, Hes-1 and Evi-1 combined with the activation of C/EBPα. The requirement of GATA-2 was not apparent since integration of GATA-2 was not detected in erythroid-like colonies by PCR. It is of note that C/EBPα is not absolutely essential for reprogramming process, since B cells could be converted into erythroid-like cells without C/EBPα activation (Supplementary Fig. 1).

3.2. GATA-1 in combination with SCL is sufficient for efficient reprogramming of B cells and C/EBPα facilitates the reprogramming

Analysis of integrated genes revealed that GATA-1-ER and SCL were commonly integrated in most of the erythroid-like colonies converted from B cells (*Supplementary* Table 3). Based on this find-

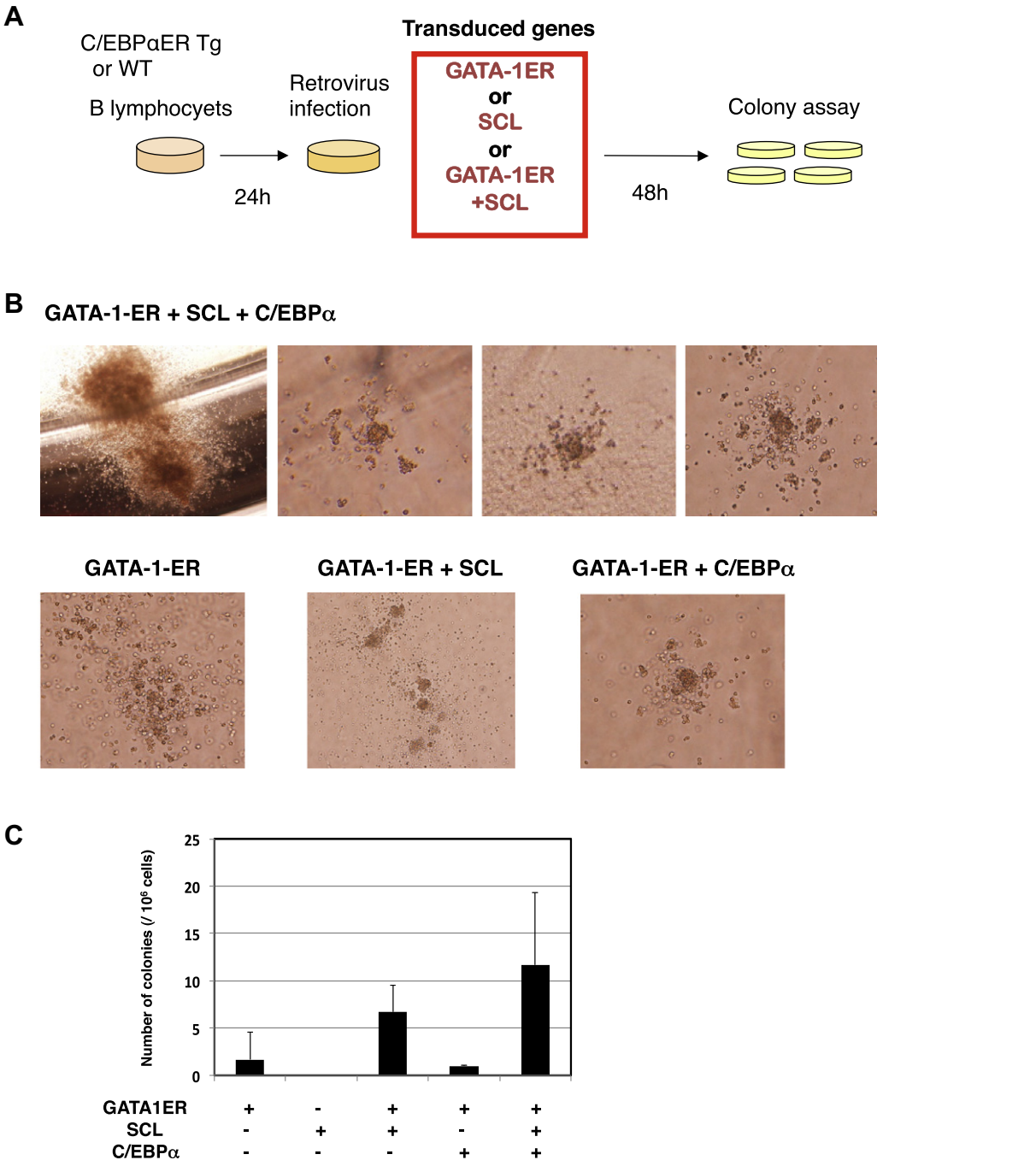


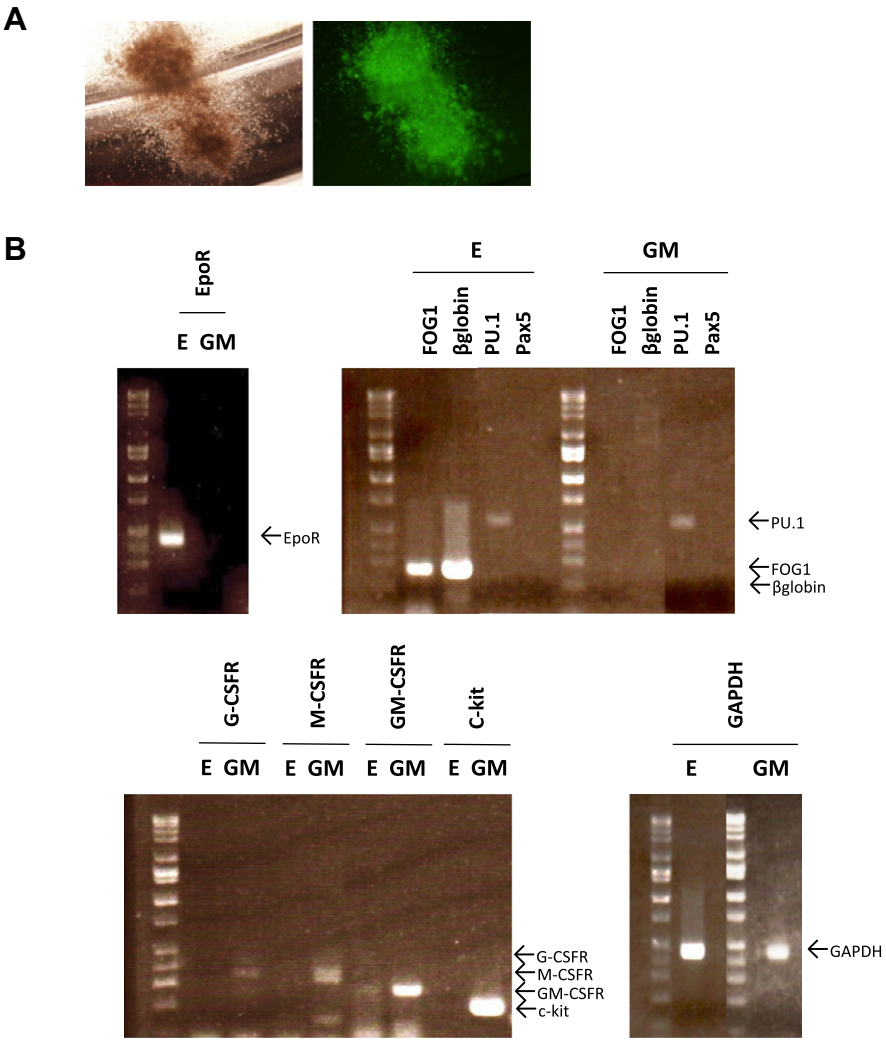
Fig. 2. Erythroid conversion of B cells by GATA1, SCL and C/EBPα. (A) Schematic illustration of retrovirus transduction to B lymphocytes from C/EBPα Tg or WT mice and colony assay. (B) Pictures of erythroid-like colonies converted by GATA-1-ER, GATA-1-ER + SCL, GATA-1-ER + C/EBPα, or GATA-1-ER + SCL + C/EBPα. (C) Numbers of erythroid-like colonies converted by various combinations of GATA1, SCL and C/EBPα (mean ± S.D., $n = 3$).

ing, we speculated that GATA-1-ER and SCL were essential for erythroid conversion. We tried to confirm this assumption by transducing GATA-1-ER or SCL separately or in combination into mature B cells from WT or C/EBPα-ER Tg mice (Fig. 2A). As shown in Fig. 2B and C, GATA-1-ER in combination with SCL efficiently converted mature B cells as compared to GATA-1-ER or SCL alone. Furthermore, co-expression of C/EBPα with GATA-1-ER and SCL enhanced the conversion efficiency approximately by twofold. These results suggest that GATA-1-ER plus SCL is sufficient to reprogram mature B cells into erythroid-like cells and co-expression of C/EBPα significantly facilitates this process.

To check whether GATA-1-ER truly mimics wild-type GATA-1, we have tried using non-inducible form of GATA-1 in the same settings, and confirmed that GATA-1 in combination with SCL was indeed sufficient for efficient reprogramming of mature B cells into erythroid lineage (Supplementary Table 4).

3.3. Gene expression of erythroid-like cells reprogrammed from B cells

We next investigated whether erythroid-like cells reprogrammed from B cells recapitulates bone a fide erythroid cells not only phenotypically but also molecularly by examining the pattern of gene expression (Fig. 3A, B and C). RT-PCR analysis revealed that reprogrammed erythroid-like colonies expressed erythrocyte-specific genes such as erythropoietin receptor (EpoR), FOG-1, and β-globin, whereas myeloid-specific genes such as gran-



C

	EpoR	FOG1	β-globin	PU.1	PAX5	G-CSFR	M-CSFR	GM-CSFR	c-Kit
E	+	+	+	+	-	-	-	-	-
GM	-	-	-	+	-	+	+	+	+

Fig. 3. Gene expression pattern of reprogrammed erythroid-like cells. (A) Morphology of representative erythroid-like colony produced from C/EBPα-ER Tg B cells by GATA-1-ER and SCL. Fluorescent image captured by fluorescent microscopy is shown on the right. (B) RT-PCR analysis for various lineage-specific genes expressed in a representative erythroid-like colony and a control GM colony. E: erythroid colony, GM: GM colony. (C) Summary of the result from RT-PCR analysis shown in (B).

ulocyte-colony stimulating factor receptor (G-CSF R), macrophage-colony stimulating factor receptor (M-CSF R), and granulocyte macrophage-colony stimulating factor receptor (GM-CSF R) were not expressed. Interestingly, PU.1, a transcription factor mainly expressed in early B and myeloid cells, was expressed in reprogrammed cells, while B-cell specific transcription factor Pax5 was not. As a control, we examined a GM colony emerged from B cells by different set of transcription factors. This clone expressed myeloid genes such as G-CSF R, M-CSF R, GM-CSF R, c-Kit and PU.1, whereas erythroid genes were not expressed. These results clearly show that erythroid-like cells reprogrammed from terminally differentiated B cells demonstrate erythroid-specific pattern of gene expression, suggesting that erythroid conversion was achieved not only phenotypically, but also at molecular levels.

4. Discussion

Generation of iPSCs from somatic cells by defined factors has enabled us to make pluripotent cells genetically identical to an

individual without raising ethical issues. Not only they serve as an ideal cell source for regenerative medicine, but also they are useful for studying tissue development and cellular differentiation, drug development, or modeling diseases. One of the major interests in the field of hematology has been the induction of mature hematopoietic cells such as red blood cells (RBCs) and platelets from iPSCs or ESCs. In fact, several investigators reported generation of RBCs or platelets in vitro from iPSCs or ESCs [12–17]. However, there are a number of problems that preclude application of induced cells or tissues from pluripotent cells. These include; (1) teratoma formation from residual undifferentiated iPSCs or ESCs when they are applied to patients, (2) time and efforts to generate iPSCs from the patients and to induce their differentiation. Direct reprogramming of somatic cells into another is an alternative, feasible approach for obtaining specific type of tissue cells [18]. By this strategy, several groups have reported successful generation of dopaminergic or glutamatergic neurons, cardiomyocytes, hepatocytes, blood progenitors from fibroblasts and β-cells from pancreatic exocrine cells [18]. These technologies are advantageous over pluripotent cells in that they theoretically have no risk of teratoma formation and they offer quicker ways to get the differentiated cells.

Within hematopoietic system, direct conversion of hematopoietic progenitors has been achieved by ectopic expression of lineage-specific transcription factors. We have previously shown that myeloid specific transcription factor C/EBPα could convert common lymphoid progenitors (CLPs) and megakaryocyte erythroid progenitors (MEPs) into myeloid lineage by using transgenic mouse model expressing inducible form of C/EBPα [9,10], and others have presented reprogramming of B and T cell progenitors into macrophages by C/EBPα [8,9]. It was also reported that GATA-1 could convert myeloid precursors and CLPs into erythroid lineage [7]. Such differentiation plasticity is not limited to hematopoietic progenitors, and in fact, some of the terminally differentiated cells that were thought to have lost differentiation plasticity were proven vulnerable to lineage converting stimuli. For example, ectopic expression of C/EBPα or PU.1 could convert terminally differentiated mature B cells into myeloid cells [8]. However, it is not clear whether mature lymphocytes could be reprogrammed into erythroid lineage or not.

In this paper, we tried to reprogram terminally differentiated mature B cells into erythroid lineage and identify factors sufficient for the reprogramming. In light of the previous report of GATA-1-mediated erythroid conversion, we assumed that GATA-1 would be the key factor to reprogram mature B cells as well. As expected, GATA-1 was essential for erythroid reprogramming of mature B cells. Interestingly however, efficient reprogramming of mature B cells required SCL and C/EBPα, whereas GATA-1 alone was sufficient for that of progenitors such as CLP and GMP. One of the reasons for this discrepancy may be the different degrees of differentiation plasticity that the cells inherently possess. In contrast to CLPs or pro-B cells, terminally differentiated mature B cells are relatively resistant to reprogramming, and their commitment to B-lineage must be unfixed by inactivation of Pax5, a critical transcription factor for B cell differentiation, for reprogramming [11]. C/EBPα was shown to suppress transcriptional activity of Pax5 and to serve as a reprogramming factor of B cells [8]. We therefore utilized C/EBPα-ER Tg mice that we previously developed in which C/EBPα can be activated conditionally by 4-HT. Our data indicated that the activation of C/EBPα indeed enhanced the reprogramming efficiency by twofold, showing that C/EBPα can serve as a facilitator of reprogramming in our system.

SCL is a member of basic helix-loop-helix (bHLH) type transcription factors, whose expression is restricted to immature hematopoietic cells and erythroid/megakaryocytic lineage in adult hematopoiesis [19,20]. An essential function of SCL in hematopoietic development such as the specification of primitive and definitive hematopoiesis was also noted by gene-knockout studies [21]. With regard to erythroid/megakaryocytic differentiation, it was reported that SCL plays an essential role in megakaryocyte and erythroid-specific gene expression programs [22]. In erythroid cells, SCL forms a multiprotein complex containing GATA-1 and their coregulators Lmo2 and Ldb1 [23,24]. This complex binds to the DNA element containing sequentially aligned E-box and GATA site, which is frequently found in the promoters of erythroid-specific genes such as EKLF [24,25]. These observations strongly suggest that GATA-1 and SCL cooperatively regulate erythroid differentiation. On the other hand, SCL regulates the expression of c-kit, a receptor for stem cell factor (SCF) [26], and SCF together with erythropoietin regulates erythroid differentiation in non-redundant fashion [27]. Taken all together, these observations clearly show a critical role of SCL in erythroid differentiation, providing a molecular basis for its synergy with GATA-1 to reprogram B cells into erythroid lineage.

We used inducible forms of C/EBPα and GATA-1 (C/EBPα-ER and GATA-1-ER, respectively) for reprogramming in an attempt to have non-inducible state serve as a negative control. Unexpectedly, however, conversion of B cells into erythroid lineage occurred in a similar frequency in either induced- or non-induced-state. It should be noted that C/EBPα-ER and GATA-1-ER proteins were considered to be functionally expressed, since the reprogramming efficiency clearly decreased without either of these factors. These results imply that the weak activities of C/EBPα or GATA-1 leaking under the non-induced state must be sufficient to help convert B cells into the erythroid lineage. It is of note that non-inducible form of GATA-1 in combination with SCL was sufficient for efficient reprogramming of mature B cells into erythroid lineage, confirming thatGATA-1-ER truly mimics wild-type GATA-1.

In summary, we showed that the combination of GATA-1, SCL and C/EBPα could directly convert terminally differentiated mature B cells into erythroid lineage. Since mature B cells can be obtained easily from peripheral blood, this study would be a valuable one-step forward in generating autologous erythrocytes by direct reprogramming.

Competing financial interests

The authors have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.019.

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